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Biochemical and Biophysical Research Communications 356 (2007) 114–117
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MD and NMR studies of α-bungarotoxin surface accessibility

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Received 16 February 2007
Available online 27 February 2007

Abstract

Protein surface accessibility represents a dimension of structural biology which has not been discussed in details so far, in spite of its fundamental role in controlling the molecular recognition process. In the present report the surface accessibility of α-bungarotoxin, a small and well characterized protein, has been investigated by analyzing its interaction with solvent and paramagnetic molecules in an integrated way. The presence of strong hydration sites, identified by a combined analysis of MD simulation and NMR results, seems to prevent the access of Gd(III)DTPA-BMA to the protein surface. On the contrary, the limited hydration of the α-bungarotoxin active site favors frequent encounters between the paramagnetic probe and the protein in the latter region. All the data obtained here for α-bungarotoxin suggest that shape and stability of the solvation shell control its surface accessibility and, hence, intermolecular interactions in a way which could be common to many other proteins.

Keywords: α-Bungarotoxin; Gd(III)DTPA-BMA; NMR; Paramagnetic probes; Protein binding sites; Protein hydration; Protein hot spots; Surface accessibility

The way specific regions of protein surfaces are available to give intermolecular interactions triggering the biological function must be encoded in their shape, atomic composition, and local dynamics. However, simple atom exposure, as determined by tertiary or quaternary structure and suitable algorithms, cannot fully account for the observed enhanced accessibility of some part of protein surfaces, commonly referred to as surface *hot spots* [1]. Thus, many experimental approaches aiming to identify accessible surface patches in proteins have been proposed [2,3]. Among them, the surface survey based on paramagnetic perturbation of NMR spectra [1] has been shown to be particularly suitable in mapping out protein *hot spots*, where protein active sites are usually located. In the past few years this technique was applied on an increasing number of protein systems, suggesting that local flexibility [4,5], transient protein–protein interactions [6], and solvent dynamics [1,2,7] may be sources of accessibility modulation.

To investigate in more details the mechanisms of protein surface accessibility, in the present study we propose a combined analysis of paramagnetic perturbations of ^{1}H–^{13}C HSQC NMR spectra and protein hydration profiles, defined by NMR measurements [8] and MD simulations.

The α-bungarotoxin (α-BTX), a small and structurally well characterized neurotoxin of *Bungarus multicinctus* snake venom, has been chosen as a model system. The α-BTX folding is characterized by three loops, referred to as finger I (residues 3–12), finger II (residues 27–39), and finger III (residues 47–57), capped by a disulfide-bond rich globular core [9]. Moreover, the interaction between α-BTX and peptides reproducing the nicotinic acetylcholine receptor has been extensively investigated, enabling an accurate identification of the toxin active site which is

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0006-291X/$ - see front matter © 2007 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2007.02.094

located in a small pocket formed by residues 7–11, 36–41, and 68–71 [10].

Materials and methods

Molecular dynamics set-up. A 16 ns MD simulation was performed in explicit solvent starting from the α-BTX solution structure (PDB code 1IK8) [9] by using the GROMACS package [11] and the GROMOS96 force field [12]. The system was simulated in the NPT ensemble by keeping constant the temperature (300 K) and pressure (1 atm); a weak coupling [13] to external heat and pressure baths was applied (relaxation times were 0.1 and 0.5 ps, respectively). The initial shortest distance between the protein and the box boundaries was 8 Å. The remaining box volume was filled with SPC type water molecules [14]. Bonds were constrained by LINCS [15] algorithm. Non-bonded interactions were accounted by using the PME method (grid spacing 1.2 Å) [16] for electrostatic contribution and cut-off (9 Å) for VDW contribution. An integration time step of 2 fs was used. The trajectory was saved every 1 ps. Since the evolution of the RMSD with time (see Supplementary Material) reaches a plateau after 2 ns, the remaining 14 ns of the MD simulation have been used for data analysis. Details on the MD trajectory analysis are reported as Supplementary Material.

Sample preparation and NMR measurements. α-BTX (Calbiochem) was used without any further manipulation. Commercial purified Gd(DTPA-BMA) (GE Healthcare) was used in the paramagnetic sample preparation. All samples were 1.0 mM in ^2H_2O. The paramagnetic sample contained Gd(DTPA-BMA) 2.0 mM.

NMR spectra, run at 303 K and pH 6.0, were obtained with a Bruker Avance 600 spectrometer. Data processing was performed with the NMRPipe software [17]. Proton and carbon chemical shifts were referenced from trimethylsilylpropionic 2,2,3,3-d_4 acid sodium salt (TSP) resonance set to 0 ppm. The experimental conditions of ^1H–^{13}C HSQC and ePHOGSY spectra have been described elsewhere [1,18].

^1H resonance assignment was carried out on the basis of previously reported NMR data (BMRB entry: 5006 and 5024) [10,19]. ^{13}C resonance assignment was obtained through conventional ^1H–^{13}C HSQC–TOCSY experiments and deposited at the BioMagResBank (entry: 15130).

Cross-peak volumes were measured with a greater that 90% confidence level using the Sparky integration tool (http://www.cgl.ucsf.edu/home/sparky/).

Details on the cross-peak attenuation (A_i) and depth index ($D_{i,r}$) calculations are reported as Supplementary Material.

Results and discussion

MD and NMR studies of α-BTX hydration

Hydration sites on the protein surface can act as a protection layer shielding the protein from its outer environment. Conversely, extended areas lacking defined and localized waters have a propensity to be in contact with dynamical solvent, becoming potential *hot spots* for protein interactions [1,2,7].

In order to establish the distribution of α-BTX hydration sites, a 16 ns MD simulation on the protein solution structure (PDB code 1IK8) [9] has been performed. The MD derived hydration sites (MDHS's) of α-BTX have been identified as local maxima in the time-averaged water density function [7]. We identified ~30 hydration sites (Fig. 1 and Supplementary Material) distributed on the surface of the toxin globular moiety, on finger I and finger III. In contrast, the toxin binding site lacks MDHS's, consistent with previous observations on the active sites anatomy [2].

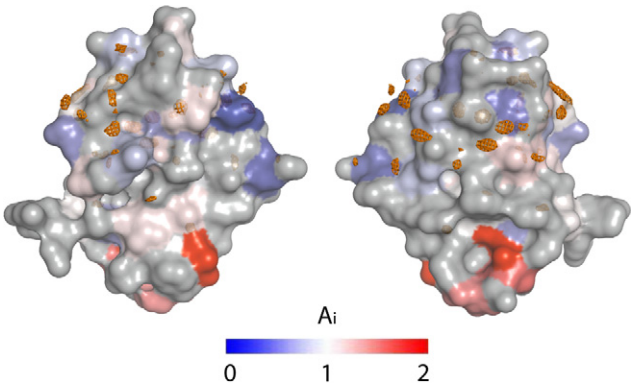

Fig. 1. Surface representation of the α-BTX showing the extent of Gd(DTPA-BMA) induced paramagnetic attenuations (A_i's). The surface is colored by CαH A_i's according to the color bar. Grid surfaces highlight the position of MDHS's showing a water density 2.3 times higher than the bulk solution.

In order to discriminate among areas characterized by similar presence of MDHS's, we integrated the static picture coming from the solvent density map with dynamic information on the α-BTX hydration shell, by estimating residence times (τ_{res}) of polar groups ↔ water interactions. Protein loci in close contact with long time resident water molecules have been found in a small hydrophilic pocket on finger I and close to a small MDHS on finger II (see Supplementary Material). Consistent to the water density analysis, no tightly bound water molecules have been observed in the toxin active site, which lays in an extended area interfacing with dynamical solvent that resembles the one found in the bulk solution.

In order to confirm the computationally derived α-BTX hydration framework, ePHOGSY spectra [20] of the toxin have been acquired. Such NMR measurement is very powerful in delineating nuclear Overhauser effects (NOE) originated by magnetization transfer from water to macromolecular nuclei. However, direct or relayed chemical exchange as well as TOCSY-type artifacts [20], can generate additional signals in the ePHOGSY spectra. Thus, a comparative search of MD and ePHOGSY convergent results can enhance the reliability of the obtained protein hydration. We found that all α-BTX atoms interacting with a water molecule exhibiting $\tau_{res} > 300$ ps also have nearby protons yielding signals in the ePHOGSY spectrum (see Supplementary Material). Thus, all the long time resident water molecules predicted by the MD simulation were experimentally observed, confirming the reliability of the overall predicted hydration pattern.

α-BTX surface accessibility

In 2D NMR spectra of proteins, neutral diffusing paramagnetic centers cause line-broadening, nuclear relaxation enhancements and, hence, cross-peak attenuations whose

extents are proportional to the local concentration of the paramagnet [1,5]. Thus, in absence of any specific interaction between paramagnetic probes and proteins, signal attenuations reflect the effective average exposure of nuclei to the bulk solution.

For typical neutral or anionic Gd(III) chelates currently used as MRI contrast agents no evidence has been found by relaxometric techniques of weak interaction with specific molecular sites [21]. Thus, we investigated the α-BTX surface accessibility by analyzing the paramagnetic attenuations (A_i) generated by Gd(DTPA-BMA) [6,22] on 39 well resolved CαH ^1H–^{13}C HSQC signals (data reported as Supplementary Material).

^1H and ^{13}C chemical shifts changes observed for the resolved α-BTX CαH correlations upon probe addition have been measured and listed in the Supplementary Material section. By inspection of the latter data, it is apparent that no correlation exists between the extent of chemical shift changes and A_i's, confirming the absence of tight interactions between α-BTX and probe.

In order to define confidence range limits for data analysis, the standard deviation (σ) between all A_i values has been calculated. Assuming that the limits of an average paramagnetic perturbation extend over ±σ around the theoretical value of the average attenuation, cross-peaks showing $1.39 \geqslant A_i \geqslant 0.61$ have been considered experiencing an average attenuation. Conversely, methyne groups showing $A_i > 1.39$ or $A_i < 0.61$ have been referred to respectively as high accessible or low accessible ones.

By analyzing Fig. 1, a good agreement between MDHS distribution and Gd(DTPA-BMA) induced paramagnetic attenuations can be inferred. In fact, strong paramagnetic effects are observed for Ala31, Ser34, Arg36, and Gly37 CαH's which, remarkably, are all located in a poorly hydrated α-BTX region. Moreover, all the α-BTX methynes experiencing limited paramagnetic attenuations are located in a wide hydrated region which is roughly opposite to the protein active site.

To discuss the observed paramagnetic effects in terms of α-BTX structural features, 3D atom depths have been quantified for all the toxin methyne groups [23]. By plotting A_i values versus atom depth indexes (D_i's) the expected agreement between paramagnetic perturbations and atom depths can be inferred (Fig. 2). In fact, methyne groups having $D_i < 0.5$, i.e. nuclei located in the protein core, exhibited low or intermediate A_i. Conversely, CαH groups with $D_i > 0.5$, i.e. atoms close to the molecular surface, showed intermediate or high accessibility to the paramagnet.

Nonetheless, anomalous A_i/D_i correlations can be observed for Ser12, Gly37, Pro49, and Lys52 CαH's. In particular, Gly37 CαH showed the highest A_i value in spite of its low surface exposure. Remarkably, Gly37 is located in the poorly hydrated binding site region, consistent with previous observations on the protein *hot spots* anatomy [1,4,18]. In contrast, the reduced probe access towards the surface exposed Ser12 CαH can be ascribed to its location in a highly hydrated region where both MDHS's and a long time resident water molecule have been found.

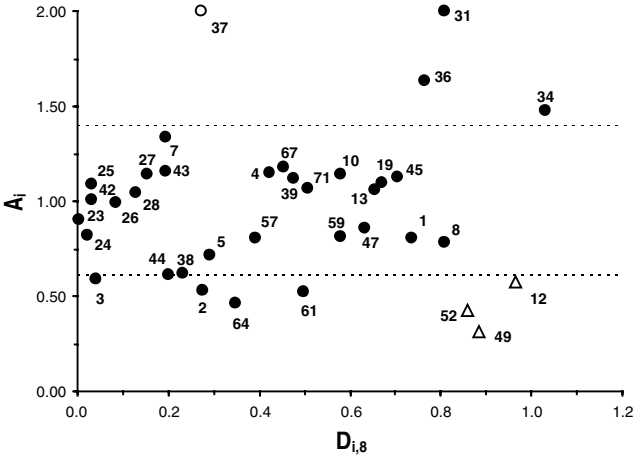

Fig. 2. Paramagnetic attenuation (A_i) versus residue depth (D_i). Open circles and triangles refer to residues showing anomalously high or low A_i values, respectively. Dashed lines refer to $A_i = 1 \pm \sigma$.

It is worth noting that Pro49 and Lys52 methyne groups, both located in a surface exposed and not hydrated α-BTX region, experienced the lowest paramagnetic effects. In fact, protein–protein interaction processes, previously observed both in the crystal state [24,25] and in solution [26], may account for this finding.

From the entire set of data presented here, several conclusions can be drawn. The surface dynamics of water molecules play a major role in driving outer molecules, including ligands and/or substrates, to the protein surface hot spots. In these critical α-BTX regions, indeed, a water layer characterized by low density and high self-diffusional features can be suggested from our MD investigation. Furthermore, poorly hydrated surface residues exhibiting low paramagnetic attenuations seem to suggest an involvement in intermolecular interactions, consistent with the recently proposed use of paramagnetic probes to define transient intermediates in protein–protein binding [27] or aggregation [6].

Thus, the combined analysis of paramagnetic perturbation profiles and hydration studies may represent a powerful tool in expanding the rational basis to understand the mechanisms of protein surface accessibility and to design protein mutants with modulated activities.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2007.02.094.

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